ON THE INFLUENCE OF PYOCYANINE ON THE RESPIRATION OF THE SEA URCHIN EGG

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Barron (1929) has demonstrated that the respiration of the unfertilized *Arbacia* and *Asterias* egg is increased by methylene blue. Runnström (1930) reported similar observations for the *Paracentrotus* egg. Runnström (1930, 1932) and Örström (1932) studied chiefly the influence of dimethylparaphenylene diamine on *Paracentrotus eggs.* This compound penetrates the eggs and causes a considerable increase of respiration both of the fertilized and the unfertilized eggs. The respiration of the eggs in the diamine solution is inhibited by cyanide and carbon monoxide. The degree of inhibition by cyanide is the same in the unfertilized and fertilized diamine eggs. The same holds true for the inhibition by CO, if the respiration is equal in the unfertilized and fertilized diamine eggs. The degree of inhibition by CO is dependent on the rate of reduction of the iron-containing enzyme of Warburg (1932). The higher the rate of reduction the more accessible is the enzyme to CO, which reacts with the reduced form of the enzyme (Warburg). Runnström inferred from his inhibition experiments that dimethylparaphenylene diamine is not auto-oxidized in the sea urchin egg but must be oxidized by the reduction of the iron-containing enzyme. Further, it was inferred that this enzyme is also present in the unfertilized egg in a reactive state, but the rate of oxidation is limited by a block in the chain of "carriers" (Keilin, 1929) which connects the iron-containing enzyme and the substrate-dehydrase system.

Barron did not find any inhibition of the respiration enhanced by methylene blue on addition of cyanide. This increase of the respiration is not due to a higher activity of the iron-containing enzyme. Methylene blue is reduced by systems in the cell which cannot react directly with molecular oxygen. It seemed to the writer of interest to try the action of pyocyanine on the respiration of the sea urchin egg. Pyocyanine belongs with respect to its oxidation reduction potential to the same range as methylene blue. As shown by Friedheim and Michaelis (1931) and further by Michaelis, Hill, and Schubert (1932),

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the pyocyanine can be oxidized or reduced in two steps, each step involving the transfer of one electron. Methylene blue, on the other hand, is oxidized or reduced in one step, each step involving the transfer of two electrons. This difference may be physiologically significant. Friedheim (1931) has shown that pyocyanine is a more effective catalyzer of some oxidations than methylene blue. Further (Friedheim, 1934), he found that the aerobic glycolysis of tumors can be decreased in presence of pyocyanine. The present writer and Michaelis (Runnström and Michaelis, 1934) have proved that the action of methylene blue and pyocyanine is different in the system hemolyzed red blood cells plus hexosephosphate. The oxygen uptake is the same with both dyestuffs, but in the presence of pyocyanine a coupling between the respiration and the synthesis of phosphate esters is induced, which does not exist or is much less conspicuous in the same system containing methylene blue.

TABLE I

Unfertilized Eggs	Control	Pyocyanine			
		0.006%	0.009%	0.012%	0.015%
cu. mm. O_2 in 150 min	30	75	84.5	93	93
Increase, *per cent*		125	180	210	210
Fertilized Eggs					
cu. mm. O_2 in 150 min	112	128	204	212	220
Increase, *per cent*		14	80	90	96

Suspension: 4.5 per cent.

The oxygen consumption was measured in the present research by Warburg's manometric method. The type of vessels used was that described by Borei (1934). It has proved to be useful for experiments with the sea urchin eggs. Into each vessel always 3 cu. cm. of the egg suspension was introduced. A 5 per cent solution of KOH was introduced into the inset as well as into one of the two side arms. The concentration of the suspension was determined by centrifuging in capillary tubes as described by Runnström (1933). The values obtained were corrected for the interstitial water by multiplication with the factor 0.64, cf. Teissier (1929). All conclusions are based on comparisons between values obtained in experiments with uniform material. Thus the exact absolute concentration of the suspensions is of minor importance.

Table I gives the evidence that the respiration is increased considerably in both fertilized and unfertilized eggs on addition of pyocyanine. The percentage increase is much higher in the unfertilized than in the

fertilized eggs. On the other hand, the absolute values of the oxygen consumption in the unfertilized eggs are lower than those of the fertilized eggs. The highest value obtained in the unfertilized eggs is still somewhat below that found in the normal fertilized eggs. The eggs can be fertilized in the solution of pyocyanine in sea water. As can be predicted from Table I, the percentage increase of the oxygen consumption is much lower than under normal conditions. At the concentration 0.006 per cent pyocyanine the increase is only 75–125 per cent, while in the control the increase amounts to 400–500 per cent. The eggs treated with pyocyanine were transferred at the end of the measurements to normal sea water. The unfertilized eggs are in no way activated and could be fertilized. The division of the fertilized eggs took place in the pyocyanine solution. In the higher concentrations (0.012–0.015 per cent) the division was, however, delayed or suppressed in spite of the increased respiration. The eggs developed after transfer to normal sea water to the pluteus stage. A certain injurious effect of

TABLE II

	Unfertilized Eggs					
Pyocyanine, *per cent*	0	0.003	0.006	0.02	0.02	0.02
HCN n	—	—	—	—	0.001	0.0025
cu. mm. O_2 in 120 min	24	36	64	82	110	128
Increase, *per cent*	—	49	167	240	360	435

the higher concentrations of pyocyanine was evident, even after transfer to normal sea water.

An *increase* of the oxygen consumption was regularly observed on addition of HCN to the unfertilized eggs in pyocyanine.

In the highest HCN concentration the oxygen consumption was equal to or somewhat higher than in the fertilized control eggs.

The same material was used for the experiments on which Tables II and III are based. The figures are thus directly comparable. The respiration of the fertilized pyocyanine eggs is decreased by addition of HCN. The residual respiration is about 70 per cent. From other experiments the residual respiration in presence of 0.0025 N HCN is known to be only about 30–35 per cent. It is easy to see that the part of the respiration induced by pyocyanine, $266 - 123 = 140$ cu. mm., is not inhibited by HCN: 30 per cent of 123 is about 40 cu. mm.; $140 + 40$ cu. mm. is equal to 180 cu. mm., which is the oxygen consumption observed in the eggs in pyocyanine plus HCN. In some

cases the residual respiration was somewhat higher than expected according to the above calculation but there is always an inhibition.

No data are available about the HCN inhibition in the unfertilized eggs of *Arbacia* in sea water without pyocyanine, but to judge from figures (Runnström, 1930) valid for the *Paracentrotus* egg, the inhibition is weak in the normal (not aged) unfertilized egg as compared with the inhibition in the fertilized egg.

The rise of the oxygen consumption of the eggs in sea water plus pyocyanine is greater than on addition of methylene blue. In one experiment the increase of respiration in unfertilized eggs was 60 per cent in presence of 0.006 per cent methylene blue, while the increase caused by 0.006 per cent pyocyanine (a concentration very nearly equimolecular to that of the methylene blue in this experiment) was 200 per cent.

At the suggestion of Dr. Alfred Mirsky, the eggs were frozen by means of solid CO_2 and ether at a temperature of about —80° C. These

Table III

	Fertilized Eggs		
Pyocyanine, *per cent*	0	0.02	0.02
HCN n	—	—	0.0025
cu. mm. O_2 in 120 min	123	266	181

Suspension: 4 per cent.

eggs were then allowed to thaw slowly at room temperature. The thawing breaks up the eggs. A part of the egg contents is dissolved in the sea water, another part remains undissolved. The thawed eggs show a certain oxygen uptake, but this is considerably enhanced by the addition of pyocyanine or methylene blue. In many experiments hexose phosphate was added, which increased the oxygen uptake 100–200 per cent. (Table IV.)

Pyocyanine gives a somewhat higher oxygen consumption than methylene blue added to the above system with hexose phosphate. In one experiment the oxygen consumption in presence of methylene blue was 97 cu. mm., while in presence of pyocyanine it was 123 cu. mm. in 120 minutes.

In the experiments cited above (Table III) it is evident that the respiration in fertilized eggs to which HCN has been added is increased by addition of pyocyanine to a level above the normal. The concentration of HCN in question blocks the mitotic process. If the eggs were brought into the HCN sea water soon after fertilization, the

division stopped in an early prophase, in the stage characterized in the living material by the appearance of the clear streak near to the nucleus. A certain increase of the volume of the nucleus takes place but the dissolution of the nuclear membrane does not occur in the presence of HCN. The block is due without any doubt to the depression of the respiration by HCN. The effect is the same in nitrogen containing traces of oxygen. In pure nitrogen even the fusion of the pronuclei and the swelling of the sperm nucleus is prevented. The normal level of oxygen consumption is more than restored by the addition of pyocyanine to the egg suspension with HCN, *yet the division is stopped at*

TABLE IV

Unfertilized eggs remained frozen during four days. After thawing the suspension was diluted 70 per cent with distilled water.[2]

Hexose monophosphate m.	—	0.012
Pyocyanine, %	0.006	0.006
cu. mm. O_2 in 130 min.	55	140

the same stage as without pyocyanine. The increased respiration is quite ineffective, when caused by the presence of pyocyanine. The eggs were also kept in pure nitrogen and it was found that the division is completely inhibited even in presence of pyocyanine, the concentration of which was varied from 0.006–0.02 per cent. The eggs were brought almost immediately after fertilization into Erlenmeyer flasks closed by rubber stoppers which were pierced by two glass tubes. Nitrogen, purified over heated reduced copper, was bubbled through the flasks. After two hours the bubbling of nitrogen was stopped and the stoppers removed. It was found that gradually the development started again. The rate of recovery is the same with and without the pyocyanine. Our results agree with those of Örström (cf., Runnström, 1933) according to which methylene blue does not promote the development of the *Paracentrotus* egg under anaerobic conditions. It seems that the opposite results reported by Rapkine (1929) are due to some technical error. The cell division can be completed only if the eggs are subject to anaerobic conditions after the dissolution of the nuclear membrane. Even then the block sets in again as soon as the segmentation furrow is formed. The respiration is necessary for the division of the sea urchin egg. This must mean that the respiration is coupled to processes in the cell necessary for the division. It is most likely that the reversible oxidation reduction systems acting as "carriers" (Keilin) bring about the coupling between the respiration and other processes in the cell. In

[2] The undiluted suspension shows a somewhat lower oxygen uptake, probably a salt effect.

the system hemolyzed blood cells plus hexose phosphate referred to above, the pyocyanine can act as a "gear" between the respiration and the synthesis of phosphate compounds. From the reported results it must be inferred, however, that in the more complicated system, the fertilized egg cell, pyocyanine cannot act as a gear between the respiration and the processes connected with the completion of the cell division.

It is quite evident from the above data that pyocyanine is auto-oxidized in the sea urchin egg. In this respect it differs from dimethyl-paraphenylene diamine but resembles methylene blue according to the experiments carried out by Barron. Friedheim (1931) reports, however, that the respiration of *Bacillus pyocyaneus* enhanced by addition of pyocyanine is decreased by CO as well as by HCN. This indicates that the pyocyanine in this case does not react or reacts slowly with the molecular oxygen. It must be oxidized by the iron-containing enzyme. This difference, as compared with the conditions in the sea urchin egg may perhaps be due to differences of pH in the interior of the cells in question.

In the unfertilized egg no part of the respiration is inhibited by HCN in presence of pyocyanine. On the contrary, the respiration is considerably enhanced by the presence of HCN. This fact cannot be explained at present but it may be remembered that HCN possibly can activate certain enzymes, the action of which increase the amount of substrate present.

In the fertilized egg the part of the respiration due to the addition of pyocyanine is not inhibited by HCN. In the fertilized egg with pyocyanine there are two auto-oxidizable systems, the iron-containing enzyme and pyocyanine. This may account for the higher absolute level of respiration observed in the fertilized eggs as compared with the unfertilized ones. In the non-aged unfertilized eggs the respiration is low. The action of the iron-containing enzyme is, according to the writer's assumption, checked by a block in the chain of "carriers." Possibly a minor part of the molecules of the iron-containing enzyme is reduced (Runnström, 1930). The possibility may also be considered that some more slowly auto-oxidizable system (like Warburg's "yellow enzyme" substrate-dehydrase) may account partly or wholly for the comparatively slow oxidations in the unfertilized eggs. Anyhow, on the addition of pyocyanine to the unfertilized eggs, the chain of oxidations apparently goes wholly over pyocyanine and not or to quite an insignificant degree over the iron-containing enzyme.

The results reported above clearly show that the dehydrase-substrate

system cannot be a limiting factor for the rate of respiration either in the fertilized or in the unfertilized egg.[3]

The experiments with dimethylparaphenylene diamine referred to above demonstrate, on the other hand, that the iron-containing enzyme is not the limiting factor. This points again to the "carriers" as the part of the respiratory mechanism which has to be made responsible for the considerable change in oxygen consumption following the activation of the egg.

This paper forms part of the work carried out by the writer in collaboration with Dr. L. Michaelis, and I wish to express my thanks to him for his generous help and valuable suggestions.

Summary

Addition of pyocyanine to a suspension of sea urchin eggs causes an increase of the respiration of the eggs. The percentage increase is higher in the unfertilized than in the fertilized eggs. The absolute values of the oxygen consumption in presence of pyocyanine are higher after than before fertilization. The respiration of the unfertilized eggs is enhanced in the presence of pyocyanine by the addition of HCN. In the fertilized eggs, on the contrary, the respiration enhanced by pyocyanine is always decreased by HCN. Pyocyanine is auto-oxidized in the sea urchin egg; the iron-containing enzyme is not involved in the oxidations induced by pyocyanine. The rate of oxidation induced by pyocyanine is somewhat higher than that of the respiration induced by methylene blue both in intact eggs and in eggs broken up by freezing and thawing. The block of division of the sea urchin egg caused by HCN or by anærobic conditions is not removed by addition of pyocyanine. In the fertilized eggs the respiration in the presence of pyocyanine and HCN can be higher than in the control; in spite of this the division stops at the same stage as when the respiration is decreased 75 per cent by

[3] Only the conditions prevailing in the egg immediately following the fertilization are considered here. During the ensuing development there is a gradual increase of respiration as found by Warburg (1926). The "increasing fraction" of the respiration is inhibited by lithium according to Lindahl (1933, 1934). The action of lithium on the increasing fraction of respiration can be accounted for by the law of mass action. Lithium possibly inhibits the formation of certain break-down products of carbohydrates by reacting with an enzyme or some part of an enzyme system. If this is true, the substrate-dehydrase system must gradually acquire the character of a limiting factor for the oxidation rate during the course of development. It may also be mentioned here that no increase of respiration was observed in living cells of baker's yeast on addition of pyocyanine or methylene blue. This holds true even if the respiration is inhibited by HCN. (Experiments carried out by M. Schüldt.) The situation in baker's yeast is very different from that in the sea urchin egg.

the addition of HCN alone. The general conclusion is drawn that neither the iron-containing enzyme nor the dehydrase-substrate system act as limiting factors for the oxidation rate in the unfertilized or newly fertilized egg.

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